Optimization of a FIA-CL Method using Luminol and Co(II) Catalyst in Presence of a Chelator for Total Antioxidant Capacity Determination

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The influence of some important experimental variables on the analytical signals of a flow injection analysis (FIA) method with chemiluminescence (CL) detection (FIA-CL) based on the use of the system luminol/Co(II)/EDTA/H_2O_2 was reported. The principle and application of the method for the determination of total antioxidant capacity (TAC) of different samples were described in several papers. However further studies on the method are necessary to avoid important errors in determinations. The following experimental variables of the FIA-CL method were studied and optimized: the flow rates of the reagents, the ratio of Co(II)/EDTA in the reagents, the injected sample volume, the concentrations of ethanol, methanol and acetone in the analyzed samples and the concentration of ethanol in the carrier. A calibration graph in different experimental conditions was traced by using caffeic acid as a standard. The optimized method was applied for total antioxidant capacity determination of several ethanolic extracts from nine Lamiaceae species.

Keywords: chemiluminescence analysis; flow injection; Co(II); luminol; total antioxidant capacity; plant extracts; caffeic acid

The oxidant-antioxidant balance is an important factor for maintaining the integrity and functionality of the tissues, organelles, organs or physiological fluids [1-3]. The reactive oxygen species (ROS) removal rate from organisms is tightly controlled by a variety of antioxidants. There is a great diversity of antioxidants: natural and synthetic, non-enzymatic and enzymatic, hydrophilic and lipophilic, endogenous and nutritional, etc. [1-4].

To determine the presence of antioxidants and their antioxidant capacity a plethora of assays have been developed: (a) spectrophotometric methods, such as 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging method [5-7, 9, 10], Folin-Ciocalteu method (FCM) for the determination of total phenols content [7-9], Trolox Equivalent Antioxidant Capacity (TEAC) [6-9], Ferric Reducing-Antioxidant Power (FRAP) method [7, 8, 11, 12], etc.; (b) methods based on luminescence: fluorescence methods, such as Oxygen Radical Absorbance Capacity, (ORAC) [9, 12-15], Total Peroxyl Radical-Trapping Antioxidant Parameter (TRAP) method [8, 10, 15], chemiluminescence (CL) methods [16-18], electro-generated chemiluminescence [19,20] and photo-chemiluminescence methods [11, 14, 21]; (c) electrochemical methods, such as amperometric [22], or voltammetric methods [23, 24]; (d) high-performance liquid chromatography (HPLC) with different detection systems, such as HPLC-CL [25] or electron spin resonance (ESR) spectroscopy [5, 20]); (f) flow injection analysis (FIA) methods with different detection methods, such as FIA-CL [26-31], multi-syringe FIA with spectrophotometric detection [32], FIA/adsorptive stripping voltammetry [33], etc. Some important reviews regarding the determination of antioxidants and antioxidant capacity are also mentioned [3, 4, 34-40].

Parejo et al [16,17] and Giokas et al [26] were the first which have described the utility of luminol/Co(II)/EDTA/H_2O_2 system for determination of the antioxidant capacity. The coupling of FIA technique with chemiluminometric detection [26, 30, 31, 41] or other detection methods [42] has the advantage of high sample throughput and a low consumption of samples. The FIA-CL method based on the use of luminol/Co(II)/EDTA/H_2O_2 system was applied with good results for the determination of total antioxidant capacity (TAC) of various samples, such as wines [31] and plant extracts [43]. However the influence of some important experimental variables of this method on the analytical signal has not yet been thoroughly investigated and reported.

The purpose of this paper was to investigate the luminol/Co(II)/EDTA/H_2O_2 system utilized in a FIA-CL assembly in order to understand the influence of the following variables: the flow rates of the reagents, the ratio of Co(II)/EDTA in the reagents, the injected sample volume, the concentrations of ethanol, methanol and acetone in the analyzed samples and the concentration of ethanol in the carrier. The optimized method was applied for the determination of TAC of some plant extracts.

Experimental part

Reagents and solutions

Boric acid, $CoCl_2.6H_2O$ (Reactivul, Bucharest), 30% H_2O_2 (w/v) (Chimopar, Bucharest), ethylenediaminetetraacetic acid (EDTA) disodium salt (Loba Chemie), 5-amino-2,3-dihydronaphtalazine-1,4-dione (luminol) (Fluka Chemie AG), caffeic acid, uric acid, gallic acid and ascorbic acid (Sigma), NaOH (Chemapol), methanol, acetone (Chemical Company, Iasi, Romania) and ethanol (S.C. P.A.M. Corporation S.R.L., Bucharest) were used.

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0.05 mol/L sodium borate buffers, *p*Hs 8.5-10 were obtained from a 0.05 mol/L boric acid solution, by adjusting the *p*H with a 10% NaOH solution. Similarly, 0.05 mol/L sodium borate buffer, *p*H 9, in 7% aqueous ethanol (v/v), was also prepared.

Co(II)/EDTA/luminol (CL reagent) solutions were prepared in 0.05 mol/L sodium borate buffer with *p*Hs 8.5, 9.0, 9.5 and 10. Co(II)/EDTA molar ratios used were 0.95; 0.9 and 0.8. EDTA concentrations used were $0.894x10^{-3}$ mol/L (for molar ratios of 0.9 and 0.95) and $1x\ 10^{-3}$ mol/L (for molar ratio of 0.8). Luminol concentration was $2.3 x\ 10^{-4}$ mol/L. The reagents were dissolved in buffer solutions in the following order: EDTA, cobalt(II) chloride and luminol.

A $2x10^{-4}$ mol/L H_2O_2 solution was prepared in a $2x10^{-4}$ mol/L EDTA solution. EDTA was employed to complex traces of metallic ions which catalytically decompose H_2O_2.

Caffeic acid solutions (2.5 - 300 μmol/L) in water, 70% acetone (v/v) or 80% aqueous ethanol (v/v) were prepared from the corresponding stock solution (10^{-3} mol/L) in water by dilution with corresponding solvents or mixtures of solvents.

All solutions were prepared in deionized water. Hydrogen peroxide working solutions were prepared on a daily basis. The other solutions were kept in the refrigerator (+4°C) for maximum three days after preparation and were brought to room temperature just before measurements. All solvents were submitted to an ultrasounds treatment (1 min) to remove air bubbles prior to analyses.

Plant extracts

The following plants belonging to the *Lamiaceae* family were used: *Hyssopus officinalis* L. (hyssop), *Lavandula angustifolia* Mill. (lavender), *Leonurus cardiaca* L. (motherwort, goosefoot), *Ocimum basilicum* L. (basil), *Origanum majorana* L. (marjoram), *Origanum vulgare* L. (oregano), *Melissa officinalis* L. (lemon balm), *Satureja hortensis* L. (summer savory) and *Thymus serpyllum* L. (creeping thyme). All were bought from local herb shops (dry vegetal material). The dried powdered plant materials were extracted in 96% ethanol (10 g dry material /100 mL solvent) for five days at room temperature, with occasional stirring. The extracts were filtered and the alcohol was rotaevaporated. The dry plant extracts were then dissolved in 70% (v/v) acetone or 80% (v/v) ethanol.

Instrumentation

The FIA-CL assembly for TAC determination is shown in figure 1 and consists of: a four-channels Gilson peristaltic pump; a Rheodyne type (model 5051) injection valve with six channels; a chemiluminometric detector specially designed for FIA analysis; a computer with a custom designed software that records the chemiluminescence signal; tygon tubes for the peristaltic pump and teflon tubes for the FIA system (i.d. = 0.8 mm). The chemiluminometric detector encloses a photomultiplier tube (PMT, power supply 1000 V), an amplifier system, a display screen and a flow cell. The flow cell was home made by spiraling a teflon tube (i.d. = 0.8 mm and length = 50 cm) in the same plane so that eight spirals were obtained. The flow cell placed on a reflecting aluminium thin foil was positioned in front of the PMT window. The carrier and the solutions of reagents were pumped though the FIA-CL assembly, according to the *Working procedure* described below. The analyzed samples were mixed with the chemiluminescence reagents right before entering the flow cell where the chemiluminescence reaction takes place.

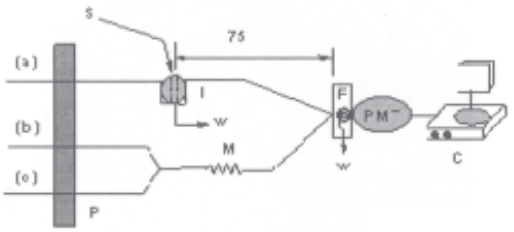

Fig. 1. FIA assembly for chemiluminescence determination [31, 43]. (a) carrier stream (0.05 mol/L sodium borate buffer, pH 9); (b) H_2O_2 solution; (c) Co(II)/EDTA/luminol solution; P: peristaltic pump; I: injection valve; M: mixing coil (i.d.=0.8 cm, L = 150 cm); F: flow cell; PMT: photomultiplier tube; C: computer; S: sample; W: waste

The principle of the method

Calculation

The principle of the method was described elsewhere [26, 31]. In brief the determination is based on the CL reaction of luminol with hydroxyl radicals, HO·, generated by a Fenton-type reaction of H_2O_2, in the presence of Co(II) ions released from a Co(II)/EDTA complex. Co(II) (as well as other transition metal cations (Fe(II), Cu(II), etc.) catalyze the decomposing of H_2O_2 according to a Fenton-type reaction:

$$Co^{2+} + H_2O_2 \rightarrow HO\cdot + HO^- + Co^{3+}$$

Co^{2+} is regenerated by the following reaction:

$$Co^{3+} + O_2^{\cdot-} \rightarrow Co^{2+} + O_2$$

The HO· radicals react very quickly with the luminol producing chemiluminescence. In the absence of a complexing agent for Co^{2+} ions a high chemiluminescence signal occurs for a short period of time. In the presence of a complexing agent (e.g. EDTA) the concentration of free Co^{2+} is very low, the chemiluminescence signal is also low, but it is more persistent [26, 31, 43].

In figure 2 the aspect of a typical chemiluminescence signal obtained at the injection of an antioxidant solution into the carrier stream is presented. I_{CL} corresponds to the value of chemiluminescence signal in the absence of an injected antioxidant. $I_{\Delta CL}$ value corresponds to the chemiluminescence decrease due to the injected antioxidant (standard or analyzed sample) solutions. In all cases, the $I_{\Delta CL}$ value of a control sample (no antioxidant, blank) was subtracted to obtain $I'_{\Delta CL}$ ($I'_{\Delta CL} = I_{\Delta CL\ sample} - I_{\Delta CLblank}$). The ratio ($I'_{\Delta CL}/I_{CL}$) x 100 was calculated.

Working procedure

The assembly presented in figure 1 was used throughout the experiments. Firstly, a 0.05 mol/L sodium borate buffer

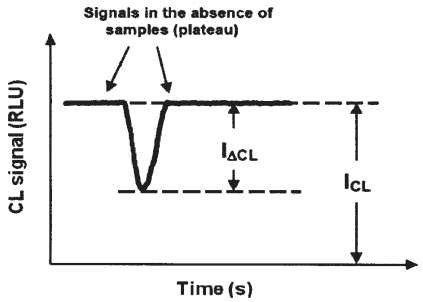

Fig. 2. The shape of the recorded FIA-CL signals [31, 43]. ΔI_{CL}: intensity of background chemiluminescence signal; $I_{\Delta CL}$: decrease of chemiluminescence signal in the presence of an antioxidant; RLU: relative chemiluminescence units

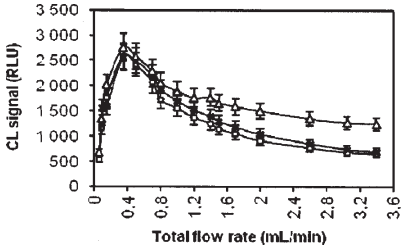

Fig. 3. Variation of the baseline chemiluminescence signal with total flow rate (mL/min) at different Co(II)/EDTA molar ratios: 0.80 (open squares); 0.90 (multiplication signs "×") and 0.95 (open triangles). EDTA concentration = 1 x 10^{-3} mol/L, luminol concentration = 2.3 x 10^{-4} mol/L, H_2O_2 concentration = 2 x 10^{-4} mol/L. RLU: relative chemiluminescence units.

solution *p*H 9, carrier, channel (a), a 2x10^{-4} mol/L H_2O_2 solution, channel (b) and a Co(II)/EDTA/luminol solution, channel (c), having Co(II)/EDTA molar ratio = 0.8 (EDTA concentration = 10^{-3} mol/L) and 2.3x10^{-4} mol/L luminol, were pumped through the system until a constant chemiluminescence signal was recorded. The antioxidant (standard/samples) solutions were subsequently injected into the carrier flow. Unless otherwise mentioned, the sample volume was 70 µL and the total flow rate was 0.45 mL/min (0.15 mL/min on each channel). The working temperature was 23 ± 1°C.

The calibration curves representing $(I'_{\Delta CL}/I_{CL})$ x100 as a function of antioxidant concentration (caffeic acid) were obtained and TAC of analyzed samples was expressed in terms of caffeic acid equivalents. Each determination was repeated at least three times and standard deviation was calculated.

Results and discussions

The effect of the total flow rate, of Co(II)/EDTA molar ratio and of injected sample volume

The effect of total flow rate on the baseline analytical response (plateau, fig. 2) at three different Co(II)/EDTA molar ratios (0.80; 0.90 and 0.95) is presented in figure 3. It was noted that at 0.36 mL/min the chemiluminescence signal was maximum for all Co(II)/EDTA molar ratios.

Nevertheless, a 0.45 mL/min total flow rate (0.15 mL/min for each channel) was selected as it afforded a reduction of the time necessary for an assay and the differences between the analytical signals obtained for total flow rates of 0.36 mL/min and of 0.45 mL/min were not significant. It was also observed that, the highest analytical signal was obtained for Co(II)/EDTA = 0.95 molar ratio. This is not surprising because at this ratio, the concentration of free Co^{2+} is the highest. Although CL signals are somewhat lower for Co(II)/EDTA = 0.8, this ratio was preferred, as higher EDTA ensures the complexation of Co^{2+} even in the presence of other trace metallic ions that can be found in the analyzed samples and that can also be complexed by EDTA. If the ratio Co(II)/EDTA is higher than 1, the method does not work.

The influence of the injected volume on the CL signal can be seen in figure 4. Volumes between 70 and 570 µL were injected. For injected volumes higher than 270µL, the values of the chemiluminescence signals tend to reach a plateau. For all the following determinations a sample injection volume of 70µL was used because at this volume the dispersion coefficient of the sample is the highest and possible interferences present in the analyzed samples have a smaller influence on the analytical signal.

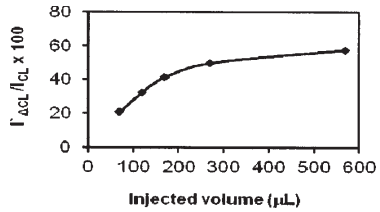

Fig. 4. Influence of the injected volume on the chemiluminescence signal. The measurements were done by using the analytical parameters described at Working Procedure (with the exception of the injected volume). Injected sample: caffeic acid, c= 4 mmol/L.

Influence of ethanol, methanol and acetone contained in the analyzed samples

Very often the plant extracts are obtained by using ethyl alcohol, methyl alcohol or acetone, and TAC determination of plant extracts are measured after solubilisation of the analyzed samples in such solvents. For this reason, the influence of these solvents on the chemiluminescence response was determined (fig. 5(a)). As can be seen from figure 5(a), ethyl and methyl alcohols in the injected samples generate a strong decrease of the chemiluminescence signal even in the absence of an antioxidant (high value for $(I'_{\Delta CL}/I_{CL})$x100). Ethyl and methyl alcohols interfere in determinations. Acetone can be used with best results as a solvent for the analyzed samples because in its presence the chemiluminescence signal is less affected (fig. 5(a)). In the case of ethanol, to diminish its influence on the recorded chemiluminescence signal, the carrier buffer solution was prepared in 7% aqueous ethanol (v/v), when a significant reduction in the ethanol influence on the CL signal could be noted (fig. 5(b)).

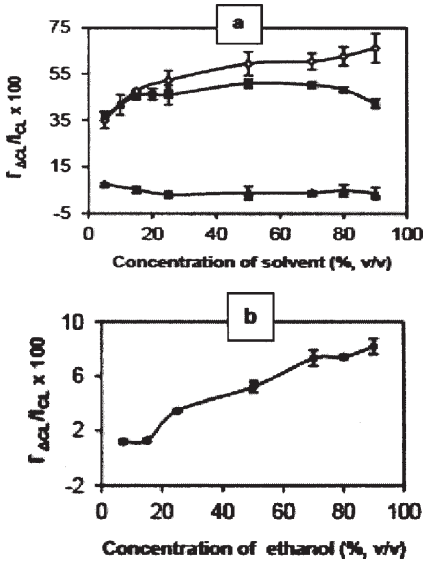

Fig. 5. Influence of ethanol, methanol and acetone contained in the analyzed samples on the chemiluminescence signal. (a) Carrier: 0.05 M, *p*H 9 borat buffer prepared in water. Ethanol: black solid squares; methanol: open diamonds; acetone: black triangles. (b) Carrier: 0.05 M, pH 9 borat buffer prepared in 7% aqueous ethanol (v/v). The measurements were done according to the Working Procedure

Calibration graphs

By representing $I'_{\Delta CL}/I_{CL}$x100 *vs.* caffeic acid concentrations, calibration curves were obtained (fig. 6(a) and 6(b)). For preparing the caffeic acid solutions three solvents were used: water, 80% aqueous ethanol (v/v) and 70% aqueous acetone (v/v).

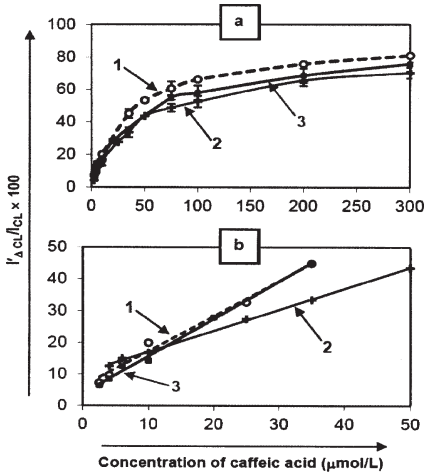

Fig. 6. Calibration curves, $I'_{\Delta CL}/I_{CL} \times 100$, as a function of caffeic acid concentration. (a) Calibration curves. (b) Linearity domain. Curve 1 (black dotted line): caffeic acid in water; curve 2 (black solid line): caffeic acid in 70% (v/v) acetone; curve 3 (gray solid line): caffeic acid in 80% (v/v) ethanol. The measurements were done according to the Working Procedure (with the exception of curve 3 for which the carrier was a buffer of 0.05 mol/L sodium borate, *p*H 9, containing 7% (v/v) ethanol)

Table 1
EQUATIONS, DETERMINATION COEFFICIENTS AND LINEAR RANGE FOR OBTAINED CALIBRATION CURVES

Curve from figure 6(b)	Equation	Linear range (μmol/L)	$(r^2/n)^b$	Solvent
(1)	$y^a = 1.099\ x + 6.33$	2.5 – 35	0.9868/6	water
(2)	$y = 0.661\ x + 10.5$	4.0 – 50	0.9988/6	70% (v/v) acetone/water
(3)	$y = 1.174\ x + 3.96$	2.5 – 35	0.9956/6	80% (v/v) ethanol/water

[a]y: $I'_{\Delta CL}/I_{CL} \times 100$; x: caffeic acid concentration (μmol/L)

[b](r^2/n): determination coefficient/number of measurements.

Table 2
THE VALUES OF TOTAL ANTIOXIDANT CAPACITY OF ANALYZED PLANT EXTRACTS

No	Herbs: Botanical name	Herbs: Common name	$I'_{\Delta CL}/I_{CL} \times 100$[a]	Dilution	TAC[b] (mg equivalents caffeic acid/g extract)
1	*Satureja hortensis* L.	savory	17.5	-	0.90
2	*Leonurus cardiaca* L.	motherwort	31.3	-	2.66
3	*Lavandula angustifolia* Mill.	lavander	36.7	-	8.46
4	*Ocimum basilicum* L.	basil	34.9 ± 1.02	1:2	13.7
5	*Melissa officinalis* L.	lemon balm	45.7 ± 2.61	1:2	24.5
6	*Hyssopus officinalis* L.	hyssop	60.3	-	32.1
7	*Thymus serpyllum* L.	creeping thyme	56.5 ± 4.35	1:2	53.4
8	*Origanum majorana* L.	marjoram	62.1	1:2	76.0
9	*Origanum vulgare* L.	oregano	58.7	1:4	125.0

[a]Values are expressed as mean of measurements (n = 2); for n = 3 it was calculated SD.

[b]TAC: total antioxidant capacity.

The equations and determination coefficients (r^2) for the linear range of each curve are shown in table 1. As can be seen from figure 6(b), the calibration curves obtained for caffeic acid in water and in 80% aqueous ethanol (v/v) carrier containing 7% (v/v) ethanol, are similar.

The developed method was used for determination of antioxidant activity of solutions (in water) of ascorbic acid, RSD % = 2.98% (20 mmol/L, n = 10), uric acid, RSD = 1.90 % (8 mmol/L, n = 10) and gallic acid, RSD = 2.41% (35 mmol/L, n = 10).

Analysis of plant extracts

In table 2 the values for the TAC determination of several plant extracts are presented. The preparation of plant extracts was described at EXPERIMENTAL and the samples were analyzed according to the described procedure. 70 μL of each plant extract were injected into the carrier stream of borate buffer prepared in 7% (v/v) ethanol, using 7% (v/v) ethanol as blank. TAC of the analyzed plant extracts varied between 0.9 and 125 mg equivalents caffeic acid/g dry extract (table 2).

The plant extracts of *Origanum vulgare, Thymus serpyllum, Hyssopus officinalis and Melissa officinalis* showed the highest TAC values, while the extracts of *L. cardiaca* and *S. hortensis* had the lowest TAC of the analyzed samples.

Conclusions

The influence of some important experimental variables on the analytical signals of a FIA-CL method based on the use of the system luminol/Co(II)/EDTA/H_2O_2 was reported. It were studied and optimized: the flow rates of the reagents, the molar ratio of Co(II)/EDTA in the reagents, the injected sample volume, the concentrations of ethanol, methanol and acetone in the analyzed samples and the concentration of ethanol in the carrier. The highest analytical signal was obtained for 0.95 Co(II)/EDTA molar ratio but it was recommended a ratio of 0.8 for further determinations. The optimum injected sample volume was of 70 μL and the total flow rate was of 4.5 mL min^{-1}. Ethyl and methyl alcohols interfere in determinations. Acetone can be used with best results as a solvent for the analyzed samples because in its presence the chemiluminescence signal is less affected. In the case of ethanol, to diminish its influence on the recorded chemiluminescence signal, the carrier buffer solution was prepared in 7% aqueous ethanol (v/v). A calibration graph in different experimental conditions was traced by using caffeic acid as a standard, the linear range varied from 2.5 to 75 μmol/L. The optimized method was applied for total antioxidant capacity determination of several ethanolic extracts from nine *Lamiaceae* species.

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